THE EMBO JOURNAL

Manuscript EMBO-2010-73946

Oncogenesis by sequestration of CBP/p300 in transcriptionally inactive hyperacetylated chromatin domains

Nicolas Reynoird, Brian Schwartz, Manuela Delvecchio, Karin Sadoul, David Meyers, Chandrani Mukherjee, Cecile Caron, Hiroshi Kimura, Sophie Rousseaux, Philip A. Cole, Daniel Panne, Christopher French, Saadi Khochbin

Corresponding author: Saadi Khochbin, INSERM/UJF U823

Review timeline:

Submission date:	02 November 2009
Editorial Decision:	12 November 2009
Additional correspondence:	02 February 2010
Additional correspondence:	03 February 2010
Resubmission:	15 February 2010
Editorial Decision:	22 March 2010
Additional correspondence:	29 March 2010
Revision received:	07 June 2010
Additional correspondences Accepted:	06 July 2010

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 12 November 2009

Thank you for submitting your manuscript for consideration by the EMBO Journal. I have now had a chance to read it carefully and to discuss it with my colleagues and I am sorry to say that we cannot offer to publish it.

We appreciate that you have investigated the molecular function of the Brd4-NUT fusion protein associated with a particular type of aggressive carcinoma. You show that NUT interacts with p300 and promotes p300 histone acetylation activity. Thus, the combination of the Brd4 acetylated histone binding bromodomain with NUT generates a feedforward loop promoting histone hyperacetylation and foci formation. You therefore propose that this would lead to large-scale epigenetic changes in expression that might account for the oncogenic function of Brd4-NUT. We find this potentially interesting, but your study does not currently address the downstream consequences of hyperacetylated histone foci formation, in terms of gene expression changes. It thus remains unclear how this change in chromatin modification and organisation would contribute to the oncogenic activity of the fusion protein. In the absence of an analysis of the functional output, I am afraid we think it highly unlikely that your manuscript would fare well under review here. Therefore, and also in the interest of saving your time, we are returning it to you with an early editorial decision, that unfortunately we can not offer to consider your study further for publication

in the EMBO Journal at this stage.

Please note that we publish only a small percentage of the many manuscripts that we receive at the EMBO Journal, and that the editors have been instructed to subject only those manuscripts to external review which are likely to receive enthusiastic responses from our reviewers and readers. I am sorry to have to disappoint you on this occasion, but I hope that this negative decision will not prevent you from considering the EMBO Journal for publication of future studies.

Yours sincerely,

Editor
The EMBO Journal

Additional correspondence 02 February 2010

In November 2009 we had submitted a manuscript to the EMBO Journal entitled "Histone hyperacetylation by a novel feed-forward mechanism induced by oncogenic BRD4-NUT" Manuscript EMBOJ-2009-73099.

In this manuscript we reported a new oncogenic mechanism by which BRD4-NUT through a feed forward loop induces and propagates hyperacetylated chromatin domains (for more details, please see our first cover letter and the manuscript attached). After careful consideration, you decided not to send the manuscript for review on the basis that the question of the "downstream consequences of the hyperacetylated histone foci formation" had not been addressed and on the "absence of an analysis of the functional output" (please see your mail dated November 12th 2009, which I have pasted below).

We have taken your points into account and we now have additional data precisely demonstrating the molecular mechanism of the oncogenic activity of BRD4-NUT fusion protein. Indeed, performing additional analyses on a BRD4-NUT expressing cell line derived from an aggressive, metastatic lung cancer arising in a young, non-smoking woman, we were able to demonstrate the molecular basis of BRD4-NUT fusion protein oncogenic activity.

We wondered if, taking into account these new data, you would now be interested in considering this manuscript and send it for review.

These additional data are as follow:

1 - BRD4-NUT titres out most of the cellular p300 into BRD4-NUT foci leading to its depletion elsewhere and a lack of its availability to direct important p300-dependent cellular functions.

2 - One of these functions is p53 acetylation and activation. We indeed show that the acetylated p53 is also sequestered into the BRD4-NUT foci leading to the shutdown of p53 regulatory circuits in these cells.

3 - A treatment of cells by the HDAC inhibitor, TSA, leads to the dispersion and disappearance of BRD4-NUT foci releasing p300, which resumes its functions. Interestingly, we have also found that TSA treatment down-regulates the total cellular amounts of BRD4-NUT, contributing to the dispersion of the foci and the restoration of p300 functions.

4 - Under these conditions (the TSA-induced disappearance of BRD4-NUT foci and the release of p300) we could demonstrate the restoration of a p53 target gene, p21 expression.

5- Most interestingly, BRD4-NUT down-regulation by specific anti-NUT siRNA also led to the release of p300 and restoration of p21 gene expression.

6 - The restoration of p53 activity induces an apoptotic cell response.

We therefore believe that these new data pinpoint a clear oncogenic mechanism by which the BRD4-NUT fusion protein mediates this aggressive and deadly carcinoma.

Taking into account the data we had previously presented, characterizing the very unique features for this unusual oncogenic protein, and these new data, we are now able to present comprehensive analyses of the molecular basis of the oncogenic activity of BRD4-NUT fusion protein.

Another important aspect of this study, which I wish to emphasise again here, is that it reveals a completely new concept in oncogenesis. Indeed, we show here how the off-context activity of a testis-specific factor in a somatic cell becomes oncogenic. Since many cancer cells aberrantly express testis-specific genes, we can propose that, at least in some cases i. e., NUT, these genes can be major contributors to malignant cell transformation.

This concept has so far been largely ignored in the field of Cancer Biology and we believe that this report would constitute a clear demonstration of this phenomenon and would hopefully shed the basis for more discoveries on the role of the off-context activity of critical testis-specific factors in somatic cancers.

Thank you for your consideration.

Additional correspondence 03 February 2010

Many thanks for your enquiry regarding a possible resubmission of your manuscript EMBOJ-2009-73099. I have now had the chance to look at it carefully, and have also discussed it with my colleagues. As I understand it, you now provide evidence that one of the consequences of the Brd4-NUT/p300 induced hyperacetylated histone foci is the recruitment and sequestration of acetylated p53. Thus, you propose that the oncogenic effects of Brd4-NUT (at least partially) due to a loss of p53 function. We can see the potential interest in this, and I recognise that this does take your study significantly further than your original submission. However, I do have a couple of concerns from what you tell me.

Firstly, you state that, by treatment with HDACi, you are able to disperse the foci, release p300 and restore p53 activity. This is in contrast to what you reported initially, and to what (to me at least) makes logical sense: namely that HDACi should lead to even more histone acetylation, spreading of the foci and recruitment/sequestration of more p300 and p53. Perhaps I have misunderstood, but I am somewhat confused by this result!

Secondly, while it does look like you have good evidence that Brd4-NUT-mediated p53 sequestration does occur, it is less clear whether you have shown that this is actually the underlying reason for the oncogenic phenotype. Given the major epigenetic changes induced, it is likely that there are significant changes in gene expression that might be the critical factors, with p53 sequestration more of a side effect. In this sense, it would be important to provide some evidence for the causal link between p53 sequestration and oncogenic potential. Your final point in your message is that "the restoration of p53 activity induces an apoptotic cell response". I'm not quite clear on what you mean by this - if you indeed show that p53 that is not recruited into the foci can reverse the transformed phenotype, this would significantly enhance the study. We would view such data as being important for us to consider the manuscript favourably.

Given these concerns (but also the potential interest), and without having actually seen the new version of the paper, I find it hard to give you a conclusive answer as to how we would view a possible resubmission. If you feel that you can address the

issues outlined above, I would encourage you to submit your manuscript formally through the system so that we can take a detailed look at it. I will then be able to give you a more informed decision, and would also be able to seek editorial input from one of our board members or other advisors if necessary. I should say that our initial handling time is very quick, so that I should be able to let you know within a few days of submission whether we would like to send the manuscript out for review. I'm sorry I can't be more definite at the moment, but I hope you understand my position. Also, please don't hesitate to get in touch if you have any further questions or comments about this.

Best wishes,

Editor
The EMBO Journal

Resubmission 15 February 2010

Following our recent exchange of mails, we are submitting our new manuscript, including additional data on the oncogenic activity of BRD4-NUT protein, for your full consideration.
First of all, I wish to thank you for your thoughtful consideration of my mail describing our new data and for the constructive questions and suggestions you made, as well as for your positive reply to re-consider our manuscript with its new data.
Also, I would like, once more, to insist on the novelty of the concepts presented here. Indeed, our data demonstrate how the off-context activity of a testis-specific regulator cooperates with a ubiquitous factor to express powerful oncogenic activities. We believe that these findings should lead the way for new developments in the understanding of the molecular basis of oncogenesis.
As described in my previous cover letter, this work presents a comprehensive molecular dissection of the activity of the BRD4-NUT fusion protein associated with a very aggressive and lethal carcinoma. We show how the fusion of BRD4 with the testis-specific factor, NUT, creates a chromatin "super-acetylator" factor and we present detailed experimental data supporting an original feed-forward mechanism for the creation of transcriptionally inactive hyperacetylated chromatin domains by BRD4-NUT.
Following your comments on the absence of a precise mechanism explaining the oncogenic activity of BRD4-NUT, we focused our efforts on the discovery of at least one mechanism that could account for oncogenesis by BRD4-NUT. This has now enabled us to produce convincing additional data.
Based on the observation of the sequestration of the majority of cellular CBP/p300 in the BRD4-NUT foci, we hypothesised that oncogenesis could arise from the depletion of CBP/p300 from its important sites of action. This led us to consider the activity of p53, which requires CBP/p300 to fully respond to critical stimuli. Following these investigations, we indeed found that p53 activity is altered in BRD4-NUT expressing cells and that it could efficiently be restored upon the down-regulation of BRD4-NUT. We also show that a TSA treatment restores p53 activity indicating that BRD4-NUT expressing cancers could be particularly sensitive to HDAC inhibitor treatments. This by itself represents a meaningful step forward in the understanding of this deadly cancer (mean survival time is about 6 months after diagnosis) and suggests new treatment strategies.
This important breakthrough came from the use of a cell line derived from an aggressive, metastatic lung cancer arising in a young, non-smoking woman, HCC2429. The cell line allowed us to first demonstrate that our major conclusions on the mechanism of BRD4-NUT action also stand in a real pathological setting. Furthermore, as mentioned above, we could demonstrate the involvement of p53 as part of the oncogenic activity of BRD4-NUT. Briefly, using p21 gene activity as an indicator of p53 function, we demonstrate a reversible inactivation of p53 in the BRD4-NUT expressing lung carcinoma cells. Interestingly a si-RNAmediated down-regulation of BRD4-NUT restores an efficient p53 activity associated with cell apoptosis.

Regarding your remarks/questions, I would also like to make the following comments.

You wrote:
Firstly, you state that, by treatment with HDACi, you are able to disperse the foci, release p300 and restore p53 activity. This is in contrast to what you reported initially, and to what (to me at least) makes logical sense: namely that HDACi should lead to even more histone acetylation, spreading of the foci and recruitment/sequestration of more p300 and p53. Perhaps I have misunderstood, but I am somewhat confused by this result!

You are absolutely right, the use of cell transfection allowed us to evidence the propagation of BRD4-NUT following a feed forward mechanism, which leads to the spreading of BRD4-NUT from the initial foci (Fig. 6d and also filmed and shown in the videos). Here the use of patientderived cells shows that the propagation of endogenous BRD4-NUT starts shortly after TSA treatment, as observed by an increase of the foci size (Fig. 6e, 30 and 60 min of treatment). However, due to the limited amounts of the endogenous BRD4-NUT expressed in these cells, and importantly due to its down-regulation after this treatment (Fig. 7c), this propagation leads to the depletion of the foci from BRD4-NUT (Fig.6e). As you correctly stated, due to massive chromatin acetylation, the residual amount of BRD4-NUT (see Fig. 7c, 300 and 480 min of TSA) becomes distributed over large genomic regions, foci disappear and p300 and acetylated p53 become evenly distributed in the nucleus (Fig. 6e and 7a).

Secondly, while it does look like you have good evidence that Brd4-NUT-mediated p53 sequestration does occur, it is less clear whether you have shown that this is actually the underlying reason for the oncogenic phenotype. Given the major epigenetic changes induced, it is likely that there are significant changes in gene expression that might be the critical factors, with p53 sequestration more of a side effect. In this sense, it would be important to provide some evidence for the causal link between p53 sequestration and oncogenic potential. Your final point in your message is that "the restoration of p53 activity induces an apoptotic cell response". I'm not quite clear on what you mean by this - if you indeed show that p53 that is not recruited into the foci can reverse the transformed phenotype, this would significantly enhance the study. We would view such data as being important for us to consider the manuscript favourably.

Your are right, BRD4-NUT could in fact, in addition to p53 inactivation, use other oncogenic mechanisms, but since p53 inactivation is by itself a strong contributor to oncogenesis and given the fact that we have clearly shown here that BRD4-NUT sequesters activated p53, I think we have found at least one of the important mechanisms responsible for oncogenesis by BRD4-NUT. This reversible inactivation of p53 is a direct consequence of the biochemical properties of the fusion protein described in detail here. First, we compared HCC2429 cells with another lung cancer cell line, A549, known to have a functional p53. Fig. 7b shows that while a genotoxic treatment efficiently induces p21 in A549 cells, there is no p21 response to this treatment in the BRD4-NUT expressing HCC2429. Interestingly, p21 gene activation occurs following the down-regulation of BRD4-NUT (Fig. 7d) or TSA treatment (Fig. 7c). These experiments clearly show that BRD4-NUT down-regulation reverts p53 inactivation, in line with the liberation of acetylated p53 and the disappearance of the foci (Fig. 7a, siRNA NUT panel).
Figure 7d also shows that BRD4-NUT down-regulation is enough to induce an apoptotic response, i. e., H2AX phosphorylation and PARP cleavage without any genotoxic treatment. This observation suggests that p53 inactivation due to BRD4-NUT allows these cells to proliferate despite the accumulation of important genotoxic stress and that the unblocking of p53 acetylation allows killing these cells.

We hope that you will now find this study complete enough to be sent to the peers for review.

2nd Editorial Decision 22 March 2010

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are enclosed. As you will see, all three reviewers express interest in your study, but all raise very significant concerns - particularly regarding the conclusiveness of the data showing that p53 sequestration is the mechanism by which Brd4-NUT induces tumorigenesis. Currently, the referees find that the evidence is indirect, and that significantly further work would be required to demonstrate that this mechanism is indeed operating and is relevant in the cancer context. Specifically, it will be critical to demonstrate conclusively that Brd4-NUT/p300 directly inhibit p53-dependent transcription - not only of p21 but also of other targets. The referees all make a number of constructive suggestions in this regard.

Given the interest expressed by the referees, I would like to invite you to revise your manuscript according to their comments. I do realise that this will entail a large amount of work, and I should stress that I would only encourage you to submit a revision if you are able to significantly strengthen the evidence to support the proposed mechanism. I therefore understand that you may wish to take your manuscript elsewhere at this point; in which case, please let us know. I also realise that the referees make a large number of suggestions, and should you wish to discuss in greater detail how to proceed with your revision, please do not hesitate to get in touch.

I should add that it is EMBO Journal policy to allow only a single round of revision. Acceptance of your manuscript will thus depend on the completeness of your responses included in the next, final version of the manuscript. When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

We generally allow three months as a standard revision time, and as a matter of policy, we do not consider any competing manuscripts published during this period as negatively impacting on the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. I realise that you may not be able to complete the necessary experiments within this three-month period, and we may be able to grant you an extension (up to a maximum of six months). Please contact us nearer the time to let us know how things are progressing and whether you expect to be able to resubmit by this deadline.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,
Editor
The EMBO Journal

———

REFEREE REPORTS:

Referee #1 (Remarks to the Author):

The manuscript by Reynoird and colleagues describes a novel mechanism downstream of the cancer-specific fusion protein Brd4-NUT, leading to massive epigenetic alterations in tumor cells. Starting from a primary observation that ectopic expression of Brd4-NUT leads to formation of hyperacetylated chromatin domains containing p300, they find that Brd4-NUT stimulates p300 HAT activity through the NUT moiety, leading to amplification of acetylated foci. Hypothesizing that sequestration of p300 may impinge on p53 transcriptional activity, they observe that in cells expressing Brd4-NUT, p21 expression is inhibited, and that knock-down of Brd4-NUT can promote its expression. Finally, they observe that treatment of cancer cells with the deacetylase inhibitor TSA results in downregulation of Brd4-NUT protein and induction of p21.
The authors describe a novel and very interesting mechanism that may lead to massive

transcriptional deregulation in tumor cells through the action of the Brd4-NUT oncogenic protein. The consequences on global transcription profile of tumor cells may derive from either inactivation of Brd4 target genes due to chromatin compaction, or from aberrant sequestration of p300 HAT in transcriptionally inactive domains. It is disappointing that this avenue and its oncogenic potential remain largely unexplored. In particular, as the authors lean towards inactivation of the p300-p53 pathway as the major oncogenic mechanism downstream of Brd4-NUT, analysis of the impact of this factor on global transcription or, at least, on p300- and p53-dependent transcription would be required. It has been demonstrated that silencing of Brd4-NUT and Brd3-NUT in NMC cell lines results in squamous differentiation and cell cycle arrest (French et al., 2008): although these phenotypes might be partly due to p53 reactivation, the involvement of p53-independent targets of p300 and of Brd4 is also likely. On the other hand, many of the authors' conclusions on the functional impact of Brd4-NUT on the p53 pathway are not fully supported by data presented, and therefore need to be clearly demonstrated.
Specific comments:
1. From Figure 1, it appears that Brd4-NUT can induce formation of chromatin foci containing acetylated H4, but not containing Brd4-NUT. How do the authors explain this? Moreover, they need to show the presence of acetylated histones in foci induced by endogenous Brd4-NUT. Does Brd3-NUT exert the same phenotype? The authors should also show localization of full-length Brd4.
2. The quantification of foci size upon p300 overexpression does not prove the establishment of a feed-forward hyperacetylation mechanism. To prove that hyperacetylated foci form through Brd4-NUT dependent stimulation of p300 HAT activity (Figure 6), the authors should analyze the effect of inhibiting p300 activity (es. with Lys-CoA) or transfecting a HAT-defective p300 mutant.
3. The proposed sequestration of p300 by Brd4-NUT would be expected to result in global gene expression changes. This should be investigated by cDNA arrays or exhaustive RT-PCR analysis of p300 transcriptional targets, to highlight other potential candidates for phenotypes such as proliferation and block of differentiation.
4. The results of the experiments performed with TSA are confusing. If TSA causes downregulation of Brd4-NUT protein, how is propagation of acetylated foci achieved? Does TSA cause downregulation of ectopic Brd4-NUT, or only of endogenous protein? Is this due to transcriptional regulation or to altered protein stability? Do other deacetylase inhibitors perform similarly?
5. There is no solid experimental evidence to sustain the claimed inactivation of p53, and the mechanism of this inactivation itself remains obscure.
First, Figure 7a does not prove that p53 is contained in BRD4-NUT foci since colocalization with BRD4-NUT is missing, and acetylated p53 normally forms discrete foci on its target genes. In addition, the effect of a second Brd4-NUT siRNA on p53 localization should be shown, as well as transfection of scrambled siRNA in a different cell line not containing BRD4-NUT.
Second, p53 does not appear to be fully sequestrated in foci, since it is still abundant throughout the nucleus (Figure 7a). How is then p53 inactivated? Is it still bound to p21 promoter and to other target genes? Lack of p21 induction may be due to p53-independent mechanisms: are other p53 targets, in particular the apoptotic ones, induced upon stress? Does Nutlin induce p21 in HCC2429 cells?
Assuming that the inhibition of p21 expression caused by BRD4-NUT relies on lack of p53 acetylation on p21 promoter, this should be investigated by performing ChIP for p300 and for acetylated p53 on p21 promoter. Moreover, treatment with TSA is normally expected to induce p21, and could occur independently of Brd4-NUT.
6. Figure 7b, d: p53 stabilization should be analyzed.
7. Figure 7d: induction of apoptosis is not convincingly shown, and should be demonstrated by either AnnexinV or TUNEL analysis. Importantly, does apoptosis depend on p53 activation?
8. Gamma-H2AX phosphorylation does not indicate apoptosis, rather ongoing DNA damage. Why is this damage induced?

Referee #2 (Remarks to the Author):

The manuscript "Oncogenesis by sequestration of CBP/p300 in transcriptionally inactive hyperacetylated chromatin domains" addresses the molecular mechanisms of oncogenic activity of BRD-NUT fusion proteins found in a subset of NUT midline carcinomas. The authors have demonstrated that overexpressed and endogenous BRD4-NUT induces the formation of hyperacetylated foci in chromatin that are not associated with active gene transcription. The authors have proposed a feed-forward mechanism for propagation of histone acetylation by BRD4-NUT.

Brd4-NUT can bind acetylated histones via its BRD4 domain. At the same time it can recruit p300 and enhance p300 HAT activity which leads to the formation of BRD4-NUT/p300 foci. The authors propose a model where BRD4-NUT inactivates p300 as a coactivator despite the increase in acetylation activity, and therefore blocks p53-dependent transcription.

This is a very interesting study both from the perspective of p300/HAT biology (addressing a longstanding mystery of p300 localization when overexpressed) and in terms of a novel p300-based anti-p53 oncogenic mechanism. A number of revisions and additional experiments will be needed to round out this study so that it may be suitable for publication in EMBO.

1) According to the proposed feed-forward mechanism for the formation of hyperacetylated foci, downregulation of p300 with siRNA should block the formation of BRD4-NUT-dependent foci. This should be shown.
2) Despite the clear difference in p21 accumulation in A549 cells and HCC2429 cells (Fig. 7), these data are not sufficient to prove that BRD4-NUT is responsible for the inhibition of p21 transcription in response to etoposide treatment. A simple experiment would be overexpression of BRD4-NUT in e.g. A549 cell line to check whether BRD4-NUT would really block p21 accumulation in response to etoposide. Additionally the effect on p21 expression should be shown at the RNA level-not just protein level to prove it is transcriptional, and additional p53 targets should be analyzed: MDM2, GADD45, PUMA/NOXA would be the minimum required.
3) There is a lack of any direct evidence that BRD4-NUT leads to p53 inactivation. The interpretation of the data does not necessarily involve p53. For example, it has been published that p21 can be induced by etoposide even in the absence of p53. So it is too preliminary to claim that "p300 sequestration into BRD4-NUT foci is the principal oncogenic mechanism leading to p53 inactivation". First, the authors need to prove that BRD4-NUT really inhibits p53-dependent transcription, e.g. by using p53 reporter constructs. Second, it also needs to be proved that p300 sequestration is the reason for p53 inactivation. One of the experiments might be overexpression of p300 mutant that doesn't bind BRD4-NUT. Will it rescue p21 stabilization by etoposide?
4) The authors demonstrate in Fig. 7d that downregulation of BRD4-NUT leads to upregulation of p21 levels, PARP cleavage and H2AX phosphorylation. First, these data are not sufficient to say that these cells undergo apoptosis. More direct assays are required- FACS profile with sub G1 accumulation+caspase 3 blot, OR caspase 3 (or annexinV) FACS analysis. Second, p21 does not induce apoptosis. Therefore, the authors need to correct this sentence: "...activation of p21 was enough to induce a spontaneous HCC2429 cell apoptosis...". It might be essential to check the expression of other p53-dependent pro-apoptotic genes (e.g., puma, noxa) as noted above in point # 2. Also-what happens with BRD4NUT siRNA when etoposide treatment is performed?
5) Is there a way to prove that TSA rescue of p21 expression is specifically antagonizing BRD4NUT activities?-as the same happens generally to p53 and p53 targets anyway without expression of the fusion protein. There may be no good way to prove such specificity, though that there is rescue of p53 activity is comforting. These possibilities of interpretation should at least be discussed.
6) Figure 7a lacks a control staining for BRD4-NUT. Therefore, it is wrong to say that "BRD4-NUT/p300 foci also contained acetylated p53"
7) Does the NUT 346-593 region have homology to other CH3 interacting proteins (E1A, E2F1, etc.)?
8) The in vitro binding of bacterial F1C to baculovirus p300 in incompletely described in Results (p.8). It should be noted that "purified p300" is actually 324-2094 truncation and the truncated version is 1045-1666. Also not clear from Materials whether the shorter version is insect cell or bacterially derived.
9) In Fig. 6a, a concurrently performed positive control with non-mutated BRD4-NUT should be shown.
10) Fig. 6c should be labeled with description of overexpressed fragments (F1C, CH3) at top of each column of photos. Some statistics on the effect on delocalization of p300 should be shown (is it 100% of cells with delocaliztion that express either fragment)? In discussion can reference Lill et al Nature 1997; 387:823 as first example of CH3 interacting protein (E1A) that disrupts p300 foci.
11) Should be noted in Intro or results that p300 has been well known to form foci only when overexpressed as first shown in Eckner et al Genes Dev 8:869 1994 (Fig. 8). Thus in Fig. 6B, cells with p300-myc transfected alone should be shown, as the foci may be the exact same size with or without BRD4NUT-and the GFP-BRD4NUT should be evaluated in a separate transfection by itself not by looking for p300 + or - cells in the same co-transfection.

Referee #3 (Remarks to the Author):

The manuscript by Reynoird et al. describes an interesting and potentially significant mechanism by which the chromosomal translocation-generated BRD4-NUT fusion protein causes oncogenesis. NUT is a tissue specific protein with unknown function and the bromodomain protein BRD4 is known to interact with acetylated histone tails and recruit P-TEFb to chromatin templates for general transcriptional elongation. In this manuscript, the authors presented a series of data indicating that the p300 HAT is a target and binding partner of the NUT protein. It is shown that p300 can be sequestrated by BRD4-NUT into hyperacetylated, nonetheless transcriptionally inactive chromatin domains that appear as discrete nuclear loci. A positive feed-forward mechanism has been proposed to explain the BRD4-NUT-mediated propagation of histone hyperacetylation. The authors further showed that the expression of BRD4-NUT and presumably its sequestration of p300 into inactive chromatin domains cause inadequate acetylation of p53, thereby disrupting the p53 tumor suppressor functions.

Overall, the paper has an interesting hypothesis and the data depicting the interaction of BRD4-NUT with p300 under transient transfection conditions and in vitro and the ability of NUT to enhance the catalytic activity of p300 are fairly convincing. However, the notion of BRD4-NUT-mediated sequestration of p300 into transcriptionally inactive chromatin domains/nucleic foci is largely based on low quality immunofluorescence staining of transfected cells. The data lack proper controls and are presented in small images with poor resolution that are difficult to evaluate and thus unconvincing. A ChIP-based assay to confirm that at genomic loci with high levels of BRD4-NUT, there is a corresponding loss of phosphorylated Pol II should be performed. As a possible explanation for BRD4-NUT-induced oncogenesis, the authors propose that the fusion protein inactivates p53 through sequestering p300, whose activity is important for p53 acetylation and activity, into transcriptionally inactive chromatin domains. While an intriguing idea, it remains to be shown whether the simple act of p300 sequestration is sufficient to cause transformation. Besides these deficiencies, the manuscript has quite a few experiments that are not well controlled or inconclusive (see below), which makes its publication premature at the current stage.

Specific comments:

Fig. 1a. Contrary to the authors' claim that BRD4-NUT and acetylated H4 are "perfectly co-localized" in discrete nuclear loci, it appears that BRD4-NUT only co-localizes with a subset of the acetylated H4 loci. In order words, some of the acetylated H4 loci apparently are not caused by BRD4-NUT overexpression. Furthermore, data in Fig. 1c also show a partial co-localization between NUT and acetylated H4, in contrast to the claim that these two proteins do not co-localize. A major problem with the experiments in Fig. 1 is that the expression levels of BRD4-NUT appears to be so much lower than those of sBRD4 and NUT, which may give rise to their distinct staining patterns. The authors need to ensure that the expression levels of the three proteins are similar in order to obtain a fair and meaningful comparison among them.

Fig. 2. The immnofluorescence staining of Pol and Ser2- and Ser5-phosphorylated CTD is either very weak or out of focus, making it difficult to evaluate the authors' claim that the BRD4-NUT loci are not associated with active gene transcription. Labeling of nascent RNA by BrUTP should be conducted in this experiment to verify the claim. A ChIP-based assay can be done on a specific gene locus to show that BRD4-NUT binding leads to a loss of Ser2- and Ser5-phosphorylated Pol II.

Fig. 3. As a rationale for placing special emphasis on the interaction of NUT with p300, the authors mentioned (without showing the actual data) that a variety of antibodies against known HATs have been used to detect their presence in the BRD4-NUT foci. These are very important data that should be shown in the paper.

Fig. 3. It would be nice to use lung cancer cell lines A549 and H1299, which were analyzed by Western blotting in Fig. 3d, as controls for immnofluorescence staining in Fig. 3e and 3g. This will allow the confirmation that the staining detected in HCC2429 cells is really due to the expression of the BRD4-NUT fusion. Secondly, it will be interesting to observe the patterns and distributions of p300 and BrUTP in cells that do not express BRD4-NUT. Do they still form discrete nuclear loci or will be distributed evenly in the entire nucleoplasm?

Fig. 4a. The domain structure in this schematic diagram is almost invisible for readers in the current manuscript. A bigger and higher resolution diagram is needed.

Fig. 5. The important notion that NUT can stimulate the HAT activity of p300 is based on experiments involving either artificial overexpression or conducted in vitro. More controls are needed to substantiate the claim and exclude the trivial possibility of a crowding effect. First, mutants of NUT that do not interact with HAT (e.g. the C-terminal domain or the F1a and F1b regions) should be co-expressed with p300 or added into in vitro reactions to show that unlike the full-length NUT or the F1c region, the mutants fail to enhance the HAT activity of p300. In addition, the HCC2429 cell line, which naturally expresses the BRD4-NUT fusion should be employed in a si/shRNA-mediated depletion to see whether the loss of BRD4-NUT will eliminate the p300 nuclear loci sequestration and reduce the overall p300 HAT activity.

Fig. 6. Panel A lacks the important control of wild-type BRD4-NUT analyzed under the same conditions. Panel B shows that the overall intensity of the BRD4-NUT signal also increases dramatically in cell co-expressing p300. Is it known whether p300 elevates the expression of BRD4-NUT? In Panel C, why would the overexpression of F1c dramatically increase the level of BRD4-NUT in HCC2429 cells? The claimed CH3-mediated dispersion of p300 in the nucleus is not very obvious and could be due to the elongated shape of that particular CH3-expressing cell. The TSA effect seen in Fig. 6d may proceed completely independent of p300 and does not provide a proof for the p300-dependent propagation of BRD4-NUT foci. This is because by inhibiting HDAC with TSA, there is more histone acetylation, which attracts more BRD4-NUT to give rise to brighter signals. Whether or not p300 is involved does not affect the outcome of this experiment. Fig. 6e and 6d seem to contradict each other and it's unclear whether the difference is simply due to different levels of BRD4-NUT expression.

Fig. 7. Do HCC2429 cells express wild-type or mutant p53 or both? How is the p53 expression level in HCC2429 cells compared to that in A549 cells before and after the treatment with etoposide? The inability to induce p21 expression in etoposide-treated HCC2429 cells could be due to either a very low level or inactive p53. This point should be clarified. The TSA-induced p21 expression in Fig. 7c could be due to a general mechanism exerting a global effect and has nothing to do with p53. Why would the introduction of a scrambled siRNA cause more p53 and p53Kac in the cell (Fig. 7a)? For all the data in Fig. 7, there is unfortunately not a single piece that demonstrates a direct involvement of p300 in the inactivation of p53 regulatory circuits by BRD4-NUT.

It would be nice to provide more background information in the Introduction section regarding the breakage points in BRD4 and NUT that are the consequences of chromosomal translocation. In addition, how the current findings relate to the known function of BRD4 in recruiting elongation factor P-TEFb to chromatin templates should be discussed.

Additional correspondence 29 March 2010

Thank you for this thoughtful analysis of our manuscript. We appreciate the careful reading and constructive advice offered by you and the reviewers.

A major and general criticism of the work is the lack of sufficient evidence to support a direct role for p300 sequestration in the BRD4- NUT foci and its consequences for p53 activity.
To remedy this, we initiated a collaboration with Phil Cole who gave us access to a specific inhibitor of p300 and its inactive control. Using these components we could comfortably confirm the direct involvement of p300 in the maintenance of BRD4-NUT foci and the down-stream events.
Additionally, using a double siRNA approach against BRD4-NUT and p53, we now show that p21 induction after BRD4-NUT knock-down is indeed p53-dependent.

We will therefore focus our attention on the other points raised by the referees and prepare a revised version of the manuscript.
Thank you very much again for your efficient handling of this manuscript.

1st Revision - authors' response 07 June 2010

Referee #1 (Remarks to the Author):

Specific comments:

1. From Figure 1, it appears that Brd4-NUT can induce formation of chromatin foci containing acetylated H4, but not containing Brd4-NUT. How do the authors explain this?

We apologize for this technical problem, which is indeed not very easy to deal with, since it is mainly due to the overall heterogeneity of the GFP fluorescence. For some foci, the GFP fluorescence needs to be enhanced to obtain a reasonable merge without saturating the already intensely fluorescent ones. Therefore when recording the GFP fluorescence (BRD4-NUT), in order to avoid the saturation of intensely fluorescent foci, some of the smaller ones with faint fluorescence disappeared. Additionally, the more intense red fluorescence of the same foci gave the impression that some hyperacetylated foci do not contain BRD4-NUT. We have tried to obtain better quality images, now shown in the new Figure 1A (and its higher magnification shown in the supplementary Figure 1).

Moreover, they need to show the presence of acetylated histones in foci induced by endogenous Brd4-NUT.

Because our anti-NUT is a rabbit antibody and most of the anti-acetylated histone antibodies are from the same species, we had to use an indirect approach based on the fact that in HCC2420 HCC2429 cells, most of the BRD4-NUT foci co-localize with p300 and most of the p300 foci colocalize with H3K18ac and H3K56ac. Consequently, the BRD4-NUT domains should therefore bear H3K18ac and H3K56ac. We have now managed to obtain several mouse monoclonal antibodies against H4K8ac, H3K14ac and H3K27ac, which allowed us to clearly show the co-localization of these marks with the BRD4-NUT foci, presented in Figure 2F (upper panels) and supplementary Figure 2D.

Does Brd3-NUT exert the same phenotype?

Our collaborator Dr. French, also co-author of the present work, is working on the molecular basis of BRD3-NUT oncogenic activity. The majority (2/3) of the NMCs involves BRD4, which justified prioritising BRD4-NUT in the present study. We believe that the molecular characterizations presented here will pave the way for other investigations including the basis of BRD3-NUT oncogenic actions. However, the complete study would require intensive molecular analyses, which would go well beyond the scope of this work.

The authors should also show localization of full-length Brd4.

The localization of full-length BRD4 is now shown (supplementary Figure 1).

2. The quantification of foci size upon p300 overexpression does not prove the establishment of a feed-forward hyperacetylation mechanism. To prove that hyperacetylated foci form through Brd4-NUT dependent stimulation of p300 HAT activity (Figure 6), the authors should analyze the effect of inhibiting p300 activity (es. with Lys-CoA) or transfecting a HAT-defective p300 mutant.

As the referee suggested, we have obtained a specific small molecule inhibitor of CBP/p300 from Dr. Phil Cole (Bowers et al., Chemistry & Biology, 2010, in press, pdf provided). The use of this inhibitor allowed us to show that upon inhibition of CBP/p300 in HCC2429 cells, both BRD4-NUT and p300 become dispersed while this effect is not observed when cells are treated with a non-active analog of this inhibitor (Figure 5D). This inhibitor has also been used to show the requirement of CBP/p300 for the activation of p53 target genes (p21, PUMA and GADD45, see below) in HCC2429 cells after BRD4-NUT knock-down (shown in Figure 6E).

3. The proposed sequestration of p300 by Brd4-NUT would be expected to result in global gene expression changes. This should be investigated by cDNA arrays or exhaustive RT-PCR

analysis of p300 transcriptional targets, to highlight other potential candidates for phenotypes such as proliferation and block of differentiation.

In order to bring additional support to the CBP/p300 sequestration hypothesis, we have now included an experiment demonstrating the role of CBP/p300 in the activation of three of the p53 target genes after the down regulation of BRD4-NUT. Indeed, Figure 6E now shows that siRNA-mediated down regulation of BRD4-NUT leads to a significant induction of p21, PUMA and GADD45 in HCC2429 cells, and that the treatment of cells with the specific CBP/p300 inhibitor suppressed this gene activation.
We hope that these new experiments will convince the referee of the strength of the p300 sequestration hypothesis. We agree with this referee that, due to this sequestration, a transcriptomic analysis would very likely show the alteration of other p300 target genes, and that taking into account the role of p300 in many cellular processes, one would expect the alteration of also other critical mechanisms. However, the scope of this work was to show that BRD4-NUT acts through CBP/p300 sequestration and our investigation of the p53 pathway has led to a clear demonstration of one of the important oncogenic consequences of this sequestration. We therefore hope that the referee will agree that, at this stage, it is not necessary to investigate the consequences of p300 sequestration on all the p300-dependent processes.

4. The results of the experiments performed with TSA are confusing. If TSA causes downregulation of Brd4-NUT protein, how is propagation of acetylated foci achieved? Does TSA cause downregulation of ectopic Brd4-NUT, or only of endogenous protein? Is this due to transcriptional regulation or to altered protein stability? Do other deacetylase inhibitors perform similarly?

We agree with the referee that the difference between endogenous BRD4-NUT and the ectopically expressed protein could have been confusing. Accordingly, we decided to remove the data regarding the effect of TSA on transiently over-expressed BRD4-NUT.
However, in order to answer the referee's question, we explain the difference, as the referee suggested, first by limited amounts of the endogenous BRD4-NUT compared to transfected protein and second by a significant down regulation of the endogenous BRD4-NUT after TSA treatment.
A western blot comparing the amounts of BRD4-NUT between transfected cells and cells expressing the endogenous BRD4-NUT showed that the ectopically expressed protein is present in a much higher quantity in transfected cells and that TSA does not seem to significantly affect the amount of transfected BRD4-NUT (not shown) probably because the over-expression exceeds the protein degradation mechanism.
Following the suggestions of this referee, first we show, using Q-RT-PCR, that the amount of BRD4-NUT mRNA is not altered in TSA-treated cells while as expected that of GADD45 accumulated (supplementary Figure 5C) and second, we used, in addition to TSA, two other classI/II HDAC inhibitors, vorinostat (SAHA), and butyrate, as well as a class III inhibitor, nicotinamide. Supplementary Figure 5D shows that while similarly to TSA, SAHA and butyrate also induced a significant down-regulation of BRD4-NUT, nicotinamide had no effect.

5. There is no solid experimental evidence to sustain the claimed inactivation of p53, and the mechanism of this inactivation itself remains obscure.
First, Figure 7a does not prove that p53 is contained in BRD4-NUT foci since colocalization with BRD4-NUT is missing, and acetylated p53 normally forms discrete foci on its target genes. In addition, the effect of a second Brd4-NUT siRNA on p53 localization should be shown, as well as transfection of scrambled siRNA in a different cell line not containing BRD4-NUT.

Since our anti-acetylated p53 and NUT antibodies were both developed in rabbit, we could not show the co-localization of acetylated p53 in the BRD4-NUT foci in HCC2429 cells. However, we made use of cell transfection experiments and are now showing in transfected cells that acetylated p53 co-localizes with GFP-BRD4-NUT foci, while total p53 is present in different parts of the nucleus (supplementary Figure 7).
The effect of the second siRNA is now shown (supplementary Figure 6A).
We did not see any effect of the scrambled siRNA on p53 background nuclear signal in A549 cells (not shown). However, in order to follow up with this and the other referees' remarks, we ectopically expressed BRD4-NUT in A549 cells and, as can be seen in Figure 6B, the presence of BRD4-NUT in these cells almost completely hindered the p53 response (p53 accumulation and p21 induction) to etoposide treatment. This experiment therefore clearly confirms the dominant role of

BRD4-NUT in neutralizing the p53 response pathway.

Second, p53 does not appear to be fully sequestrated in foci, since it is still abundant throughout the nucleus (Figure 7a). How is then p53 inactivated? Is it still bound to p21 promoter and to other target genes? Lack of p21 induction may be due to p53-independent mechanisms: are other p53 targets, in particular the apoptotic ones, induced upon stress? Does Nutlin induce p21 in HCC2429 cells?

We now show that two other p53 target genes, PUMA and GADD45 become activated upon BRD4-NUT down-regulation (Fig. 6E).
We purchased Nutlin and following the reviewer's suggestion, both HCC2429 and A549 cells were treated. As shown in the Figure (referee 1 only suppl data), although Nutlin efficiently induces p21 accumulation in A549 cells, it has no effect on p21 in the HCC2429 cells. This experiment shows that in HCC2429, although p53 is wt (we have sequenced the cDNA from these cells), its level is higher compared to A549 cells and that nutlin does not induce a further accumulation of p53. Since we have no explanation for this observation and can only speculate, we have decided not to include this figure into the manuscript.

Assuming that the inhibition of p21 expression caused by BRD4-NUT relies on lack of p53 acetylation on p21 promoter, this should be investigated by performing ChIP for p300 and for acetylated p53 on p21 promoter. Moreover, treatment with TSA is normally expected to induce p21, and could occur independently of Brd4-NUT.

We agree with this referee on the fact that TSA could induce p21 independently of p53. The experiments investigating the effect of BRD4-NUT on the p53 response pathway are now shown in the new Figure 6.
These experiments show that, in the absence of a TSA treatment, p21 is activated after the siRNA-mediated down-regulation of BRD4-NUT (Fig. 6C). In order to prove that this activation is really p53-dependent, in a double knock-down experiment, we have also down-regulated p53 itself in addition to BRD4-NUT and, under these conditions, the induction of p21 does not occur (Fig. 6D). Additionally, as mentioned above, we also show that the ectopic expression of BRD4-NUT in A549 cells severely interferes with the activation of the p53 response after a genotoxic treatment (Fig. 6B). Finally, as suggested by this referee, we have performed ChIP in HCC2429 cells for the presence of p53, acetylated p53 and p300 on the p21 promoter (before and after BRD4-NUT down-regulation). Figure 6F shows that the down-regulation of BRD4-NUT is associated with the recruitment of acetylated p53 and p300 onto the p21 promoter.
We believe that these results definitely show that one of the important consequences of the BRD4-NUT activity is the neutralization of p53 regulatory circuits.

6. Figure 7b, d: p53 stabilization should be analyzed.

This is now shown in the new Figure 6A (an improved version of the ex-Fig. 7b).

7. Figure 7d: induction of apoptosis is not convincingly shown, and should be demonstrated by either AnnexinV or TUNEL analysis. Importantly, does apoptosis depend on p53 activation?

An anti-activated caspase 3 now convincingly shows, by western and FACS measurements, the occurrence of cell apoptosis after BRD4-NUT down-regulation (Figure 6C and supplementary Figure 6B). We could also show that the down-regulation of both BRD4-NUT and p53 prevents the activation of caspase 3 normally observed when BRD4-NUT is down regulated alone (Figure 6D) proving therefore its dependance on p53.

8. Gamma-H2AX phosphorylation does not indicate apoptosis, rather ongoing DNA damage. Why is this damage induced?

We agree and have now replaced gamma-H2AX by activated caspase 3. One possible explanation could be that the sudden release of the compact BRD4-NUT associated chromatin after its down regulation may be associated to some topological constraints, DNA damage and H2AX phosphorylation.

Referee #2 (Remarks to the Author):

1) According to the proposed feed-forward mechanism for the formation of hyperacetylated foci, downregulation of p300 with siRNA should block the formation of BRD4-NUT-dependent foci. This should be shown.

Based on the suggestion of this referee, we used a specific small molecule inhibitor of p300, as well as its inactive counterpart, obtained through collaboration with Dr. Phil Cole (Bowers et al., Chemistry & Biology, 2010, in press, pdf provided). The use of this inhibitor allowed us to obtain the exact result predicted by the referee: the BRD4-NUT foci disappeared. This is now shown in Figure 5D (see also Figure 6E).

2) Despite the clear difference in p21 accumulation in A549 cells and HCC2429 cells (Fig. 7), these data are not sufficient to prove that BRD4-NUT is responsible for the inhibition of p21 transcription in response to etoposide treatment. A simple experiment would be overexpression of BRD4-NUT in e.g. A549 cell line to check whether BRD4-NUT would really block p21 accumulation in response to etoposide. Additionally the effect on p21 expression should be shown at the RNA level-not just protein level to prove it is transcriptional, and additional p53 targets should be analyzed: MDM2, GADD45, PUMA/NOXA would be the minimum required.

The experiments suggested by the referee have been performed and the results are now shown in Figure 6B and 6E.
They indeed show, as predicted, that when BRD4-NUT is ectopically expressed in A549 cells, it almost completely hinders the p53 response to etoposide treatment. This experiment therefore clearly confirms the dominant role of BRD4-NUT in neutralizing the p53 response pathway.
Q-RT-PCRs were used to show that the down-regulation of BRD4-NUT results in the induction of p21, PUMA and GADD45 at the mRNA levels and interestingly, a treatment of cells with the CBP/p300 inhibitor blocked this activation (Figure 6E).

3) There is a lack of any direct evidence that BRD4-NUT leads to p53 inactivation. The interpretation of the data does not necessarily involve p53. For example, it has been published that p21 can be induced by etoposide even in the absence of p53. So it is too preliminary to claim that "p300 sequestration into BRD4-NUT foci is the principal oncogenic mechanism leading to p53 inactivation". First, the authors need to prove that BRD4-NUT really inhibits p53-dependent transcription, e.g. by using p53 reporter constructs. Second, it also needs to be proved that p300 sequestration is the reason for p53 inactivation. One of the experiments might be overexpression of p300 mutant that doesn't bind BRD4-NUT. Will it rescue p21 stabilization by etoposide?

In order to show the direct involvement of p53 we have used a double siRNA approach to knock-down both BRD4-NUT and p53, and this allowed us now to show that p21 induction is indeed p53-dependent (Figure 6D).
Following the referee's suggestion, we have also used a p53-reporter construct and shown that BRD4-NUT actually represses the p53-dependent activity of the reporter (please see supplementary Figure 7B).

4) The authors demonstrate in Fig. 7d that downregulation of BRD4-NUT leads to upregulation of p21 levels, PARP cleavage and H2AX phosphorylation. First, these data are not sufficient to say that these cells undergo apoptosis. More direct assays are required- FACS profile with sub G1 accumulation+caspase 3 blot, OR or caspase 3 (or annexinV) FACS analysis. Second, p21 does not induce apoptosis. Therefore, the authors need to correct this sentence: "...activation of p21 was enough to induce a spontaneous HCC2429 cell apoptosis...". It might be essential to check the expression of other p53-dependent pro-apoptotic genes (e.g., puma, noxa) as noted above in point # 2. Also-what happens with BRD4NUT siRNA when etoposide treatment is performed?

The misleading sentence regarding p21 and apoptosis has now been changed. As suggested by the referee, we have used an anti-activated caspase 3 antibody to show its clear accumulation after BRD4-NUT knock-down by western and FACS measurement as requested by the referee (Figure 6C, 6D and supplementary Figure 6B).
The expression of other p53 target genes, including the pro-apoptotic gene PUMA has now been tested and the results show a significant induction of this gene after BRD4-NUT down-regulation

(Figure 6E).
Finally, we could show an enhanced HCC2429 apoptotic cell response to etoposide after BRD4-NUT knock-down (supplementary Figure 6B).

5) Is there a way to prove that TSA rescue of p21 expression is specifically antagonizing BRD4NUT activities?-as the same happens generally to p53 and p53 targets anyway without expression of the fusion protein. There may be no good way to prove such specificity, though that there is rescue of p53 activity is comforting. These possibilities of interpretation should at least be discussed.

This is an interesting point, which at least partly deals with p300 sequestration and p53 inactivation. In fact, the sequestration of CBP/p300 by BRD4-NUT appears to be similar to the previously reported sequestration of CBP/p300 in some neurodegenerative diseases, which could be rescued by HDAC inhibitor treatment (see for instance, McCampbell et al., Nature, 2001). The major difference with the poly-Q-protein aggregates is that BRD4-NUT specifically recruits and sequestrates p300 while the poly-Q aggregates imprison many essential cellular components leading to cell degeneration rather than oncogenesis.
Here, our aims were to dissect the oncogenic activity of BRD4-NUT and to test our working hypotheses. Additionally, these investigations also show that BRD4-NUT is a "drugable" target and that new drugs such as an anti-BRD4 bromodomain small molecule inhibitor could also be a choice leading to foci dispersion or yet better and more specific molecules could be those targeting NUT and disrupting NUT-p300 interaction.

6) Figure 7a lacks a control staining for BRD4-NUT. Therefore, it is wrong to say that "BRD4-NUT/p300 foci also contained acetylated p53"

The referee is right. Because our antibodies are both from rabbit the colocalization was not possible. To show the presence of acetylated p53 in BRD4-NUT foci, we had to use a transfection-based approach. Supplementary Figure 7A now shows that, as expected, acetylated p53 accumulates in the GFP-BRD4-NUT foci.

7) Does the NUT 346-593 region have homology to other CH3 interacting proteins (E1A, E2F1, etc.)?
The NUT 346-593 region, although it is the most conserved part of NUT when compared with mouse and Rat Nut, has no significant sequence homology with other known domains.

8) The in vitro binding of bacterial F1c to baculovirus p300 in incompletely described in Results (p.8). It should be noted that "purified p300" is actually 324-2094 truncation and the truncated version is 1045-1666. Also not clear from Materials whether the shorter version is insect cell or bacterially derived.

We apologize for not having clearly given this information in the previous version. The complete information has now been added. Also it is now mentioned clearly that all the p300 species used are expressed in insect cells and purified from the corresponding extracts.

9) In Fig. 6a, a concurrently performed positive control with non-mutated BRD4-NUT should be shown.

This has now been added (Figure 5A).

10) Fig. 6c should be labeled with description of overexpressed fragments (F1C, CH3) at top of each column of photos. Some statistics on the effect on delocalization of p300 should be shown (is it 100% of cells with delocaliztion that express either fragment)? In discussion can reference Lill et al Nature 1997; 387:823 as first example of CH3 interacting protein (E1A) that disrupts p300 foci.

All the referee's remarks have been taken into account. Nearly 100% of the cells expressing either fragments show this delocalisation. This is now mentioned in the legend of the Figu.

11) Should be noted in Intro or results that p300 has been well known to form foci only when

overexpressed as first shown in Eckner et al Genes Dev 8:869 1994 (Fig. 8). Thus in Fig. 6B, cells with p300-myc transfected alone should be shown, as the foci may be the exact same size with or without BRD4NUT-and the GFP-BRD4NUT should be evaluated in a separate transfection by itself not by looking for p300 + or - cells in the same co-transfection.

All the referee's remarks have been taken into account. Data of Eckner et al., are now discussed in the results section and Myc-p300 alone is now shown (Figure 5B).

Referee #3 (Remarks to the Author):

Specific comments:

1 - Fig. 1a. Contrary to the authors' claim that BRD4-NUT and acetylated H4 are "perfectly co-localized" in discrete nuclear loci, it appears that BRD4-NUT only co-localizes with a subset of the acetylated H4 loci. In order words, some of the acetylated H4 loci apparently are not caused by BRD4-NUT overexpression. Furthermore, data in Fig. 1c also show a partial co-localization between NUT and acetylated H4, in contrast to the claim that these two proteins do not co-localize. A major problem with the experiments in Fig. 1 is that the expression levels of BRD4-NUT appears to be so much lower than those of sBRD4 and NUT, which may give rise to their distinct staining patterns. The authors need to ensure that the expression levels of the three proteins are similar in order to obtain a fair and meaningful comparison among them.

We apologize for this technical problem, which is indeed not very easy to deal with, since it is mainly due to the overall heterogeneity of the GFP fluorescence. For some foci, the GFP fluorescence needs to be enhanced to obtain a reasonable merge without saturating the already intensely fluorescent ones. Therefore when recording the GFP fluorescence (BRD4-NUT), in order to avoid the saturation of intensely fluorescent foci, some of the smaller ones with faint fluorescence disappeared. Additionally, the more intense red fluorescence of the same foci gave the impression that some hyperacetylated foci do not contain BRD4-NUT. We have tried to obtain better quality images, now shown in the new Figure 1A (and its higher magnification shown in the supplementary Figure 1).

Fig. 1C shows a very diffuse distribution of NUT all over the nucleus, with almost no distinguishable discrete domains.
Fig. 2B shows that when expressed in cells, Ha-NUT and Ha-BRD4-NUT show comparable levels of expression.

2 - Fig. 2. The immnofluorescence staining of Pol and Ser2- and Ser5-phosphorylated CTD is either very weak or out of focus, making it difficult to evaluate the authors' claim that the BRD4-NUT loci are not associated with active gene transcription. Labeling of nascent RNA by BrUTP should be conducted in this experiment to verify the claim. A ChIP-based assay can be done on a specific gene locus to show that BRD4-NUT binding leads to a loss of Ser2- and Ser5-phosphorylated Pol II.

Following the referee's suggestion we have performed the requested experiment of BrUTP pulse labelling in both HCC2429 (expressing the endogenous BRD4-NUT) and A549 (not expressing the fusion protein). Please see the new Figure 2G.
Unfortunately, we could not undertake a ChIP-based assay, as the referee suggested, since no specific target of BRD4-NUT is known.
We have also tried to improve the quality of the images now shown in supplementary Figure 2B, which had previously suffered (loss of resolution) during the transfer from our image software to pdf. We apologize for this.

Fig. 3. As a rationale for placing special emphasis on the interaction of NUT with p300, the authors mentioned (without showing the actual data) that a variety of antibodies against known HATs have been used to detect their presence in the BRD4-NUT foci. These are very important data that should be shown in the paper.

These experiments are now shown as supplementary Figure 3.

Fig. 3. It would be nice to use lung cancer cell lines A549 and H1299, which were analyzed by Western blotting in Fig. 3d, as controls for immnofluorescence staining in Fig. 3e and 3g. This will allow the confirmation that the staining detected in HCC2429 cells is really due to the expression of the BRD4-NUT fusion. Secondly, it will be interesting to observe the patterns and distributions of p300 and BrUTP in cells that do not express BRD4-NUT. Do they still form discrete nuclear loci or will be distributed evenly in the entire nucleoplasm?

We now show the pattern of BrUTP incorporation in both HCC24 and A549 cells as requested by the referee (Fig. 2G). This Figure also shows that in A549 cells the anti-NUT antibody does not detect anything similar to the BRD4-NUT foci detected in HCC2429 cells.
The pattern of p300 in a non BRD4-NUT-exressing cell is shown in Fig. 2A where two Cos7 cells are shown one expressing BRD4-NUT. This Figure shows that the pattern of p300 differs between the two cells. Additionally, the new Figure 5 and supplementary Figure S5 show several examples of p300 distribution depending on the activity of CBP/p300 or the presence of BRD4-NUT in HCC2429 cells.

Fig. 4a. The domain structure in this schematic diagram is almost invisible for readers in the current manuscript. A bigger and higher resolution diagram is needed.

We apologize for this low-resolution figure. A bigger diagram is now shown (new Figure 3A).

Fig. 5. The important notion that NUT can stimulate the HAT activity of p300 is based on experiments involving either artificial overexpression or conducted in vitro. More controls are needed to substantiate the claim and exclude the trivial possibility of a crowding effect. First, mutants of NUT that do not interact with HAT (e.g. the C-terminal domain or the F1a and F1b regions) should be co-expressed with p300 or added into in vitro reactions to show that unlike the full-length NUT or the F1c region, the mutants fail to enhance the HAT activity of p300. In addition, the HCC2429 cell line, which naturally expresses the BRD4-NUT fusion should be employed in a si/shRNA-mediated depletion to see whether the loss of BRD4-NUT will eliminate the p300 nuclear loci sequestration and reduce the overall p300 HAT activity.

As suggested by the referee, a non-p300 interacting fragment of NUT was used and the results obtained show that there is no stimulation of p300 HAT activity (supplementary Figure 4). Additionally, regarding the second remark of the referee, we have obtained a specific small molecule inhibitor of CBP/p300 from Dr. Phil Cole (Bowers et al., Chemistry & Biology, 2010, in press, pdf provided). The use of this inhibitor allow us now to show that, upon the inhibition of CBP/p300, both BRD4-NUT and p300 become dispersed while this effect is not observed when cells are treated with the non-active analog of this inhibitor (Fig. 5D).

Fig. 6. Panel A lacks the important control of wild-type BRD4-NUT analyzed under the same conditions.

This control has now been re-introduced (new Fig. 5A).

Panel B shows that the overall intensity of the BRD4-NUT signal also increases dramatically in cell co-expressing p300. Is it known whether p300 elevates the expression of BRD4-NUT?

This could be due to the accumulation of the smaller BRD4-NUT foci into larger ones where BRD4-NUT could be protected against degradation and stabilized. The HDACi-dependent down-regulation of BRD4-NUT could in fact be due to the dispersion of BRD4-NUT.

In Panel C, why would the overexpression of F1c dramatically increase the level of BRD4-NUT in HCC2429 cells?

In fact, the anti-NUT antibody recognizes the F1c fragment. Therefore, in the cell expressing F1c we observe both F1c and BRD4-NUT. We apologize for the confusion. This was mentioned in the legend of the Figure and is now also mentioned in the text. The important point we wanted to show here is the dispersion of p300 in these cells.

The claimed CH3-mediated dispersion of p300 in the nucleus is not very obvious and could be due

to the elongated shape of that particular CH3-expressing cell.

Fig 5C: Another field is now shown.

The TSA effect seen in Fig. 6d may proceed completely independent of p300 and does not provide a proof for the p300-dependent propagation of BRD4-NUT foci. This is because by inhibiting HDAC with TSA, there is more histone acetylation, which attracts more BRD4-NUT to give rise to brighter signals. Whether or not p300 is involved does not affect the outcome of this experiment. Fig. 6e and 6d seem to contradict each other and it's unclear whether the difference is simply due to different levels of BRD4-NUT expression.

We fully agree with this referee. We were certainly not clear enough in presenting the rationale of these experiments. The TSA experiment was not used as a proof of the involvement of p300 in the propagation of BRD4-NUT, but to support the idea that an increase in chromatin acetylation should lead to the propagation of BRD4-NUT all over chromatin and to the disappearance of discrete foci. We have now attempted to clarify this point in the text.
It is clear that cells ectopically expressing GFP-BRD4-NUT produce much higher amounts of the protein compared to the HCC2429 cells where, additionally, TSA treatment induces a down regulation of the protein. Since this experiment on the ectopically expressed BRD4-NUT is confusing and does not bring critical information, we decided to omit these results and describe and discuss the effect of HDAC inhibitors only on HCC2429 cells (this Figure and some additional data are now shown in supplementary Figure S5).

Fig. 7. Do HCC2429 cells express wild-type or mutant p53 or both?

We have cloned and sequenced p53 mRNA from these cells and the sequence shows only the wild type transcript (not shown).

How is the p53 expression level in HCC2429 cells compared to that in A549 cells before and after the treatment with etoposide? The inability to induce p21 expression in etoposide-treated HCC2429 cells could be due to either a very low level or inactive p53. This point should be clarified.

The new Figure 6A now shows the level of p53 in these cell lines before and after etoposide treatment. Indeed, for unknown reasons, the basal level of p53 is higher in HCC2429 cells than in A549 cells and in any case this indicates that the difference in p21 accumulation is not due to low levels of p53 in HCC2429 cells.

The TSA-induced p21 expression in Fig. 7c could be due to a general mechanism exerting a global effect and has nothing to do with p53.

The referee is right. The role of p53 is now specifically shown in the experiments involving BRD4-NUT down-regulation. We have now completed these experiments with a double knock-down approach using anti-NUT and anti-p53 siRNAs. The new Fig. 6D shows that knocking down p53 prevents p21 induction showing therefore the requirement of p53 for p21 induction after BRD4-NUT knock-down.

Why would the introduction of a scrambled siRNA cause more p53 and p53Kac in the cell (Fig. 7a)?

The referee is right, there is in fact some cell-to-cell variability and the cell shown in the "no-treatment" panel was not representative of the general p53-related fluorescence. The mean situation resembles more the two cells shown in the scrambled panel. A new Figure is now shown (supplementary Fig. 6).

For all the data in Fig. 7, there is unfortunately not a single piece that demonstrates a direct involvement of p300 in the inactivation of p53 regulatory circuits by BRD4-NUT.

We have now added new data showing that siRNA-mediated down regulation of BRD4-NUT leads to a significant induction, in addition of p21, of two other p53 targets, PUMA and GADD45 in HCC2429 cells, and that the treatment of cells with the specific small molecule CBP/p300 inhibitor, strongly represses this gene activation. These results are now shown in Fig. 6E. Additionally, as

mentioned above, the inhibition of CBP/p300 leads to the dispersion of BRD4-NUT foci in the HCC2429 cells (Fig. 5D).

It would be nice to provide more background information in the Introduction section regarding the breakage points in BRD4 and NUT that are the consequences of chromosomal translocation. In addition, how the current findings relate to the known function of BRD4 in recruiting elongation factor P-TEFb to chromatin templates should be discussed.

The information on the breakage point is now given in the introduction and a discussion has now been added on BRD4 in the discussion section.

Additional correspondence 25 June 2010

Many thanks for submitting the revised version of your manuscript EMBOJ-2010-73946. It has now been seen again by the original referees 1 and 2, whose comments are enclosed below. As you will see, both find the manuscript to be substantially improved and have no further concerns. I am therefore pleased to be able to tell you that we will be able to accept your manuscript for publication in the EMBO Journal.

However, before we can do so, I do have one issue with Figure 3 that I need to ask for some clarification on. In panels B, C, E and F, there are thin lines between lanes: does this indicate that you have spliced together lanes that were initially separated? This is acceptable (assuming that all lanes do come from the same gel), but obviously not ideal - particularly in the case of Figure 3C, where each lane is separate. Minimally, you need to state clearly in the figure legend what has been done here; ideally, I would encourage you to consider running a new gel with the samples next to each other, and replacing the relevant panel(s). I also note that in the bottom part of panel F, the lanes marked ZZ-HA and TAZ2-HA have apparently been spliced together without this being marked.

In this situation, I also need to ask you to send me the original scans of all the relevant blots for this figure, so that we can see how the panels have been assembled. These will be published as supplementary material.

Can I therefore ask you to get back to me and let me know how these panels were made, to change the figure as necessary, and to add an explanatory note in the figure legend? You can send all the files (new figure, text file and original scans) by email, and we will upload them into the system. Once this has been sorted out, we should then be able to accept your manuscript!

Many thanks for your cooperation with this.

Referee 1:

The authors have made sufficient attempts at answering the questions raised, and now the paper has been significantly improved. The data are now sufficient to sustain the proposed mechanism, although I feel that further dissection of the mechanism of p53 inactivation would reveal additional levels of complexity. In summary, I now recommend publication of the manuscript in its present form.

Referee 2:

The authors have responded to all the comments and requested revisions in a more than adequate fashion. The paper is appropriate for publication in EMBO in present form.

Additional correspondence 25 June 2010

Thank you very much for your efficient handling of our manuscript and for the very constructive interactions.

Please find below the clarifications of the points you have raised.

1 - In panels B, C, E and F, there are thin lines between lanes: does this indicate that you have spliced together lanes that were initially separated?

As you can see in the original scans (attached), in all the panels (B, C, E and F), all the samples were run together.
However, to increase the clarity of the presentation and make the figure more compact, in the case of the HA-tagged proteins (anti-HA), which are of different sizes, we chose to only show the corresponding areas of the films.

The original scans of B, C, E and F show how the panels were mounted.

In the case of Figure 3F, we forgot to draw the lines which have now been added.

In the case of the bottom panel, it is a montage corresponding to the same experiment run on two gels. As you can see in the Figure 3F-1, due to a transfer problem the input for ZZ fragment was not detected.

The two samples corresponding to ZZ and TAZ2 were therefore run on another gel. This time the input of the ZZ fragment was clearly visible (Fig. 3F-2). The signal corresponding to the TAZ2 fragment showed that the gel was comparable to the first one, which allowed us to use this ZZ input to mount the final Figure.

We are now sending a new Figure 3 with its legend to better highlight these points. In this new Figure, a line was added only where the HA- tagged fragments are shown.

Please let us know whether these explanations are clear enough for you and whether the way we have presented the data appears acceptable.

Many thanks again for your help.

Additional correspondence 28 June 2010

Many thanks for your explanations as to the assembly of Figure 3 and for sending me the original scans. Most of it looks fine, but I still have a couple of remaining concerns. Firstly, in Figure 3E, can you please clarify why you have two bands corresponding to p300 Myc, one of which is cut out in the final panel? More seriously, I'm sorry to say that I don't think we can proceed to publication with Figure 3F. You state that the figure is a montage of two gels, done this way because of transfer problems with the original experiment. However, you show some lanes from this "problematic" blot - including lanes with no signal - which could call into question the validity of the results here. Moreover, the interpretation of the results requires the direct comparison of lanes from two independent experiments, which is not really acceptable. While we are not in any way doubting the results of this experiment, I am afraid we have to insist on your repeating this experiment so that all the samples are run on the same gel.

Please can you just get back to me about this, and also give me some kind of timeline as to when you expect to be able to repeat this one experiment. Once you have done this, you can just send me a new version of Figure 3 and its accompanying legend; I will also need to see the original scans of the new experiment. I hope this will not be too problematic or time-consuming for you, and I'm sure you understand our need to be vigilant about these kinds of things!

Many thanks for your cooperation with this.

Additional correspondence 29 June 2010

Thank you very much again for the careful consideration of our manuscript.

We perfectly understand your concern and there is no problem in repeating the experiment shown in Fig. 3F.

In fact, as I am sure you can imagine, this experiment, showing a GST pull-down had to be performed several times with each GST-fused fragment in the set-up phase (allowing to calibrate the amount of GST- fusion to use). We therefore knew the results of the interactions with confidence and a final experiment was performed to prepare the figure, where this technical problem occurred, so another gel was run with the same samples to perform the final montage. The presence of common samples on both gels allowed merging them.

It is however understandable that you prefer to see the results of the samples run on a same gel. This will be done and a new Figure 3 will be sent to you. We believe that the whole experiment should be doable in around 10 days.

Figure 3F shows the results of another pull-down experiment. As you know under pull-down conditions, due to the addition of bacterially expressed GST proteins, it is very difficult to avoid some degradation of large proteins such as p300.

The lower bands are therefore the results of this degradation. In fact we did not "choose" to show only one band, but the full-length band to illustrate the results of the interactions. Moreover, as you can see, one of these degradation bands is visible in the figure (lane 6). I would like to insist that the point here was to show the capture of full-length p300 of p300 CH3 domain by NUT F1c fragment, and that the presence of some p300 degradation products, by no means modifies the conclusions, as one has to compare the amount of this full-length band in the input to what is captured by GST and GST-F1c.

Please let me know if you would like us to show larger fields.

Thank you very much again for your understanding.

Additional correspondence 29 June 2010

Great - thanks for the explanation, and I'm pleased to hear that it won't be aproblem to repeat the experiment. As for the degradation bands, I think it should be fine to leave the panel as it is - as you say, it in no way changes theinterpretation of the data.

I look forward to receiving the final updated figure and associated text, and – once again - many thanks for your cooperation with this!

Additional correspondence 04 July 2010

Thank you very much again for giving us the opportunity to improve our manuscript. Nicolas has worked hard last week and repeated the experiment shown in Figure 3F.
The panel has now been replaced and a new Figure 3 has been prepared (attached)

This time, as you can see on the original scans (also attached), all the samples were run on the same gel.

We hope that you will now find this new figure acceptable.

Acceptance 06 July 2010

Many thanks for dealing with this so quickly and for sending the new figure - everything looks fine now. We will upload these new files into our system, and should then be able to accept your manuscript for publication without further delay.

I really appreciate your cooperation with this, and am happy that we can publish this nice piece of work!

